

Full Length Research Paper

The protective effect of 7-difluoromethylgenistein on oxidative stress injury induced by hydrogen peroxide in PC12 cell line

Xing Feng*, Mei-Ling Tao, Xiao-Hua Fu, Hong-Lin Xiang and Jian-Guo Cao

College of Medicine, Hunan Normal University, Changsha, Hunan 410006, P.R. China.

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7-Difluoromethylgenistein (FMG), prepared by the difluoromethylation and alkylation of genistein, is an active new chemical entity. The protective effect of FMG on PC12 cell injury by oxidative stress was studied, and its molecular mechanism of action was also investigated. This paper demonstrated that pretreatment with FMG could improve cell growth and proliferation in a concentration dependent manner, reduce the release of lactate dehydrogenase (LDH), suppresses the H₂O₂-induced apoptosis of PC12 cells, up-regulate expression of phosphorylated ERK1/2 and suppress activation of p38 in H₂O₂-induced PC12 cells. Therefore, these results suggested that FMG possessed the protective activity of PC12 cells injury by H₂O₂ *in vitro*. The protective activity is associated with up-regulating the expression of phosphorylation ERK1/2 and suppressing the activation of p38.

Key words: Genistein, 7- difluoromethylgenistein, PC12 cell, oxidative stress, protective effect.

INTRODUCTION

Oxidative stress is a pathological process of cell toxicity resulted from the loss of balance of oxygen free radical production and scavenging, or excessive intake of exogenous oxidants leading to reactive oxygen species (ROS) accumulation in the body (Simonian and Coyle, 1996). Studies have shown that many pathogenesis of the central nervous system diseases are closely related to oxygen free radicals, such as cerebral ischemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis (Weber, 1994). Therefore, developing the effective agents for anti-oxidative damage to delay the processes of these diseases and aging, and then exploring their protection of cellular mechanisms have become important research topic in the medical field.

Genistein (Figure 1) is an isoavonoid compound derived from soy products. Its chemical structure is similar to estradiol and it could play a role similar to that

of estrogen (Kwon et al., 2007; Huang et al., 2007). Genistein has many significant biological activities, such as anti-tumor, anti-virus, anti-fungal, bactericidal, anti-oxidation, anti-mutagenic, anti-hypertensive, anti-inflammatory and anti-proliferative (Knight and Eden, 1996). One of the mechanisms in disease prevention is the strong anti-oxidative stress (Kapiotis et al., 1997; Carbajal and Schaeffer, 1998; Somjen et al., 2001). However, the absorption of genistein in the gastrointestinal tract is poor, resulting in low biological activity (Kroom et al., 2004).

To overcome this problem, synthesizing some novel chemical entities based on genistein might be an effective process. It is well known that the introduction of the CHF₂ group into organic molecules can change their chemical and physical properties (Abazinge et al., 2009). Then a series of genistein derivatives was designed and synthesized (Fu et al., 2008).

After the introduction of two fluoro-methyl-(CF₂) into genistein, 7 - difluoromethyl -5 - alkoxy - series of derivatives of genistein was used as target molecules to obtain nine difluoromethyl-derivatives of genistein for the

*Corresponding author. E-mail: fengxing01@hotmail.com, Tel: +86 731 88912473, Fax: +86 731 88912417.

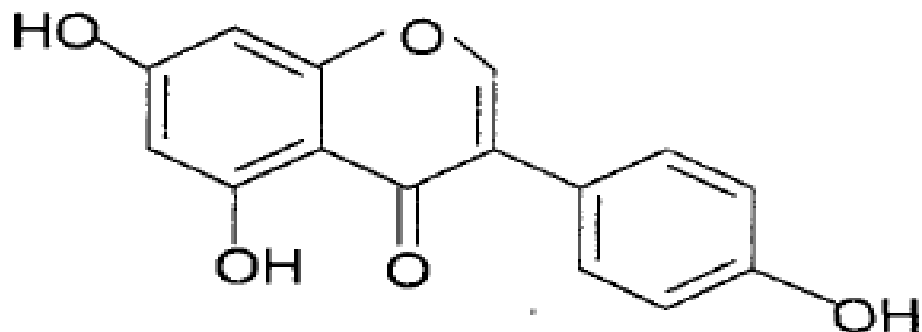
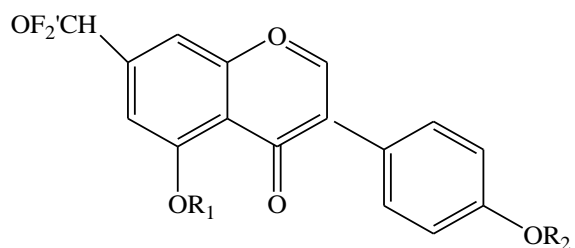


Figure 1. The structure of genistein.



Compound	R1	R2
FMG	H	H
GEN-1	CH ₃	CH ₃
GEN-2	CH ₃ CH ₂	CH ₃ CH ₂
GEN-3	n-C ₃ H ₇	n-C ₃ H ₇
GEN-4	C ₆ H ₅ CH ₂	C ₆ H ₅ CH ₂
GEN-5	n-C ₇ H ₁₅	n-C ₇ H ₁₅
GEN-6	n-C ₈ H ₁₇	n-C ₈ H ₁₇
GEN-7	n-C ₁₀ H ₂₁	n-C ₁₀ H ₂₁
GEN-8	ISO-C ₄ H ₉	ISO-C ₄ H ₉

Figure 2. The structure of FMG and other derivatives of genistein.

test articles (Figure 2). Alkoxy-based may effectively improve bioactivity of compounds (Bois et al., 1999). Then we sieved an active new chemical entity. 7-difluoromethylgenistein (FMG) was sieved from these test articles which had the most effective protection of nerve cells injury by oxidative stress and vascular endothelial cell damages caused by hydrogen peroxide (Fu et al., 2008). However, the effect of FMG on PC12 cells injury by oxidative stress and its mechanism were

still unknown.

In this study, the effect of FMG on PC12 cells and its molecular mechanism was investigated. Lactate dehydrogenase (LDH) activity of PC12 cells in different treatment group was compared. The cell apoptosis was detected by using flow cytometry (FCM) and the cell proliferation determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Finally, the expression level of p-ERK1/2 and p38 in PC12 cells were

investigated by western blotting method.

MATERIALS AND METHODS

Reagents

FMG (98% pure) and other genistein derivatives were synthesized as reported in our laboratory (Fu et al., 2008). Genistein, MTT, Tris and Australia phenol blue were purchased from Sigma, USA. RPMI-1640 medium and Dulbecco's minimum essential medium (DMEM) were obtained from Gibco, USA. Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Company (Hangzhou, China). Hydrogen peroxide (H_2O_2) was obtained from Guangdong Jinhua Chemical Factory (Guangzhou, China). Trypsin, Tween20, and dimethyl sulfoxide (DMSO) were purchased from Amresco, USA. Rabbit anti-human p-ERK1/2 multi-resistance, Rabbit polyclonal antibody anti-human p38, Mouse anti-human p-actin monoclonal antibody, Horseradish peroxidase labeled goat anti-mouse IgG antibody, Horseradish peroxidase HRP goat anti-rabbit IgG antibody were purchased from Wuhan Boster Biological Engineering Company (Wuhan, China). BCA Protein Assay Kit was purchased from Hyclone-Pierce, USA. Glycine, Propylene Thalidomide, and A Bis, Thalidomide were purchased from Novon, USA. SDS, persulfate amine, TEMED, and DTT were obtained from Huamei Bio-Engineering Company, China.

Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) were originated from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37°C in a 5% CO_2 incubator. When cells were near 80% confluence, new media with newborn calf serum were added before the compounds treatment. We used 40 μM H_2O_2 to establish an oxidative-stress injury model *in vitro*, and sieved an active new chemical entity using the model. We adopted this model as a reference methodology in our study. H_2O_2 was added in final concentrations ranging from 5 to 80 μM in the pilot study, and the concentration (40 μM) was selected by determining dose-response curves. When needed, cells were incubated for 30 min with genistein, derivatives of genistein, FMG and then exposed to 40 μM H_2O_2 for 24 h.

Detection of LDH activity of PC12 cells

When PC12 cells were close to 80% confluence, new media with newborn calf serum was added before the compounds treatment. Cells were treated with genistein, derivatives of genistein, FMG for 30 min followed by the addition of H_2O_2 to a final concentration of 40 μM , and incubated for 24 h. The medium was collected to a 1 mL Eppendorf tube, then the LDH activity of PC12 cells was detected by multifunction biochemistry analyser.

FCM with propidium iodide (PI) staining

The PC12 cells were treated in the same way as described above. Cells were collected, digested with 0.25% trypsin and made into a single cell suspension by RPMI-1640 medium supplemented with 10% newborn calf serum. The single cell suspension was centrifuged at 1000 rp/min for 5 min at 4°C. The supernatant was removed, washed with cold phosphate buffered saline (PBS), centrifuged at 1000 rp/min for 5 min at 4°C. Cells were resuspended as a single cell suspension with 50 μl PBS, fixed with

1 ml of cold 70% ethanol, stained with PI and cell apoptosis was detected by using FCM.

MTT assay

Logarithmic growth phase cells were seeded in 96-well plates at a density of 5×10^3 /ml. Cells were cultured for 24 h at 37°C with 5% CO_2 , treated in the same way as described above. Each group was set up for three parallel holes. Cells were cultured for 24 h, followed by incubation with 0.5 mg/mL MTT, 200 μl serum-free medium for 4 h. Finally, 100 μl of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of enzyme-linked immunosorbent instrument.

Relative cell proliferation inhibition rate (IR) = $(1 - \text{average } A_{570} \text{ of the experimental group} / \text{average } A_{570} \text{ of the control group}) \times 100\%$.

Western Blot analysis

When PC12 cells were near 80% confluence, new media with newborn calf serum were added before the compounds treatment. Cells were treated with genistein (10 μM), FMG (1.0 and 10 μM) for 30 min followed by the addition of H_2O_2 to a final concentration of 40 μM . Cells were treated with H_2O_2 for 0, 4, 8 and 24 h, respectively, lysed by cell lysis, and then centrifuged at 12000 rp/min for 10 min at 4°C. The extracted protein sample (40 μg total protein/lane) was added in the same volume of sample buffer solution and subjected to denaturation at 100°C for 5 min, then electrophoresed on 100 g/L sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and finally transferred onto polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was treated with Tris buffered saline Tween-20 (TBST) containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with p-ERK1/2, the first antibodies p38 (1:200 dilution), respectively, at 4°C overnight. After being washed with TBST for 10 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. The membrane was then washed three times for 10 min each with TBST. Fluorescence was produced from Solution A and B containing a chemiluminescence-generating chemical. The results were analyzed with Image analyzer (SPSS 16.0 software package) and the product of area and optical density was expressed as integral absorbance.

Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. Data were represented as mean \pm SD. The means of multiple groups were compared with one-way analysis of variance (ANOVA), after the equal check of variance, and the two-two comparisons among the means were performed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

FMG decreased the LDH release of PC12 cells

As shown in Figure 3, after PC12 cells were treated with H_2O_2 (40 μM) for 24 h, the release of LDH was significantly increased from (26.00 ± 1.00) U/ml to (92.67 ± 5.86) U/ml. The LDH release Results showed that FMG could effectively reduce the release of LDH H_2O_2 -induced

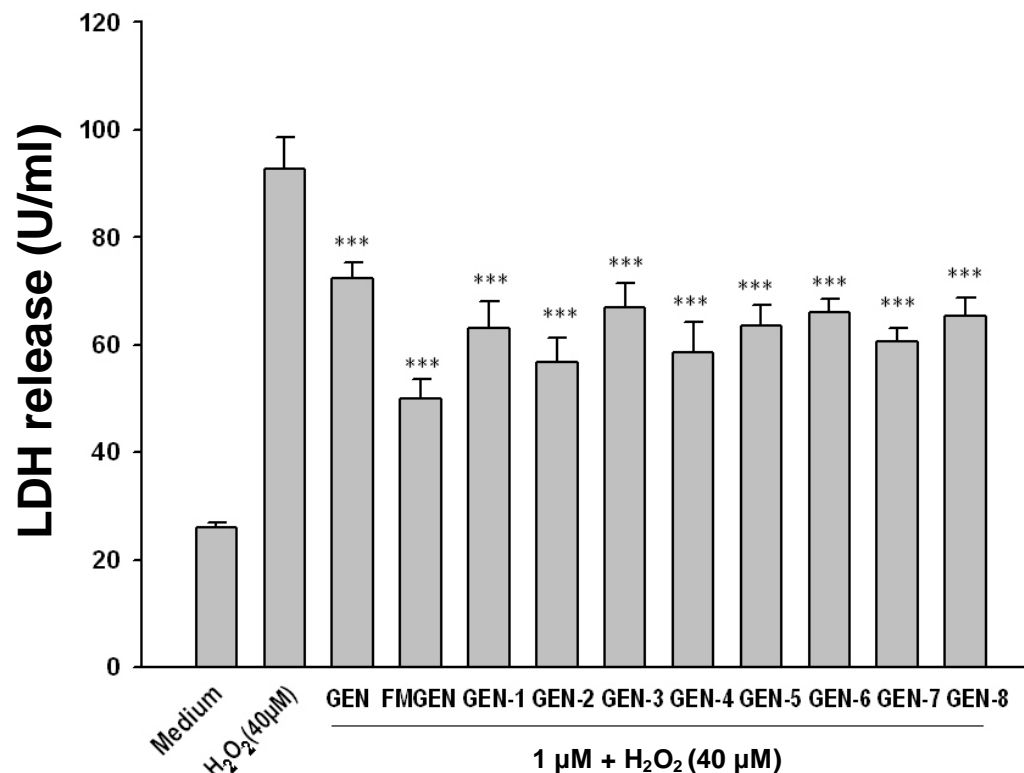


Figure 3. PC12 cells were seeded and treated as described in the materials and methods. The culture medium from each treatment was collected and the LDH activity was analyzed. Data shown here represent the average of three experiments. The concentrations of all the derivatives were 1.0 μmol / L, *** P < 0.001 versus H₂O₂ injury group, ** P < 0. 01 versus H₂O₂ injury group.

PC12 cells than the lead compound genistein in a concentration dependent manner.

FMG reduced hydrogen peroxide-induced apoptosis in PC12 cells

FCM with PI staining showed that the apoptosis rate of PC12 cell line treated with 40 μmmol/L H₂O₂ for 24 h was 34.07±3.44%, which was significantly higher than that of medium group (0.89±0.06%). When 0.1, 1.0 and 10 μM of FMG were added to the assay, cell apoptosis rate was reduced to 13.83±0.42, 5.99±0.46 and 2.89±0.37% in a concentration dependent manner. FMG could suppress the H₂O₂ -induced apoptosis of PC12 cells among all the derivatives. The results also showed that FMG could effectively reduce the H₂O₂ -induced apoptosis of PC12 cells than the lead compound, genistein (Figure 4).

FMG reduced hydrogen peroxide-induced proliferation inhibition rate in PC12 cells

The MTT assay demonstrated that the inhibitory rate of cells was significantly increased to 36.14% after PC12

cells were treated with H₂O₂. When PC12 cells were treated with 0.1, 1.0 and 10 μmol/L FMG, the apoptosis rate of cells were reduced to 33.32, 13.80 and 1.99%. The results showed that FMG could significantly decrease H₂O₂-induced inhibition of PC12 cells in a concentration dependent manner than the lead compound genistein. FMG was the most potent among all the derivatives (Figure 5).

FMG down-regulated the expression of phosphorylation of ERK1/2 and p38 protein in hydrogen peroxide-induced PC12 cells

Western blot analysis demonstrated that the expression level of ERK1/2 phosphorylation and p38 protein phosphorylation were up-regulated after PC12 cells were treated with H₂O₂. When 1.0 and 10 μM FMG were added to the assay, the expression level of phosphorylation ERK1/2 increased to 107.6±1.5% and 111.8±2.4%, respectively. Results showed that FMG could strongly activate the H₂O₂-induced expression of phosphorylation ERK1/2 of PC12 cells than genistein (Figure 6). However, When 1.0 and 10 μmol/L FMG were added to the assay, the expression level of phosphorylation p38 decreased to

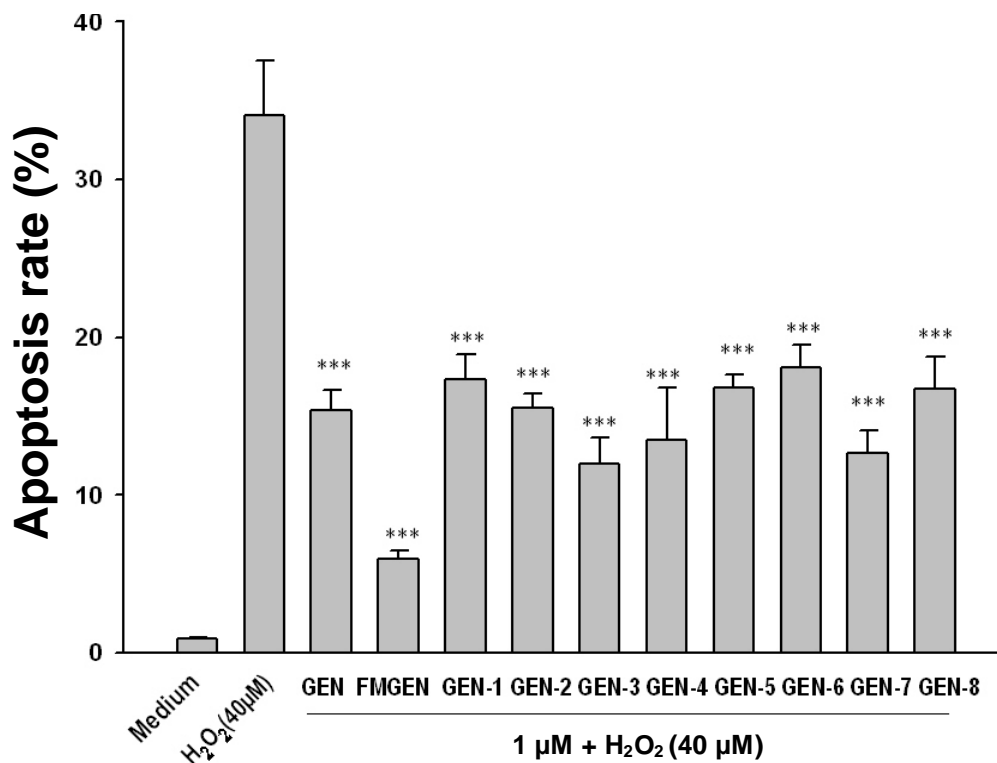


Figure 4. FCM has shown that all genistein derivatives can decrease apoptosis rate of H₂O₂ – induced PC12 cells. Among them, FMG was the most potent. Data shown here represent the average of three experiments. The concentrations of all the derivatives were 1.0 μmol / L. *** P < 0.001 versus H₂O₂ injury group, ** P < 0.01 versus H₂O₂ injury group.

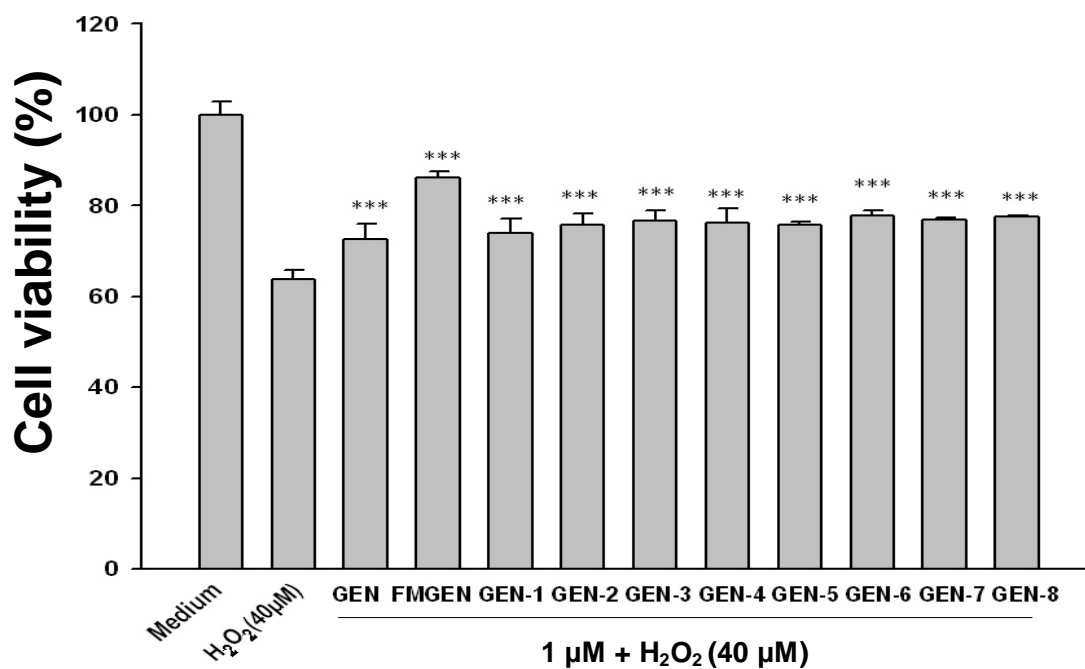


Figure 5. MTT assay has shown that all genistein derivatives can increase proliferation rate of H₂O₂ – induced PC12 cells. Among them, FMG was the most potent. Data shown here represent the average of three experiments. The concentrations of all the derivatives were 1.0 μmol / L. *** P < 0.001 versus H₂O₂ injury group, ** P < 0.01 versus H₂O₂ injury group.

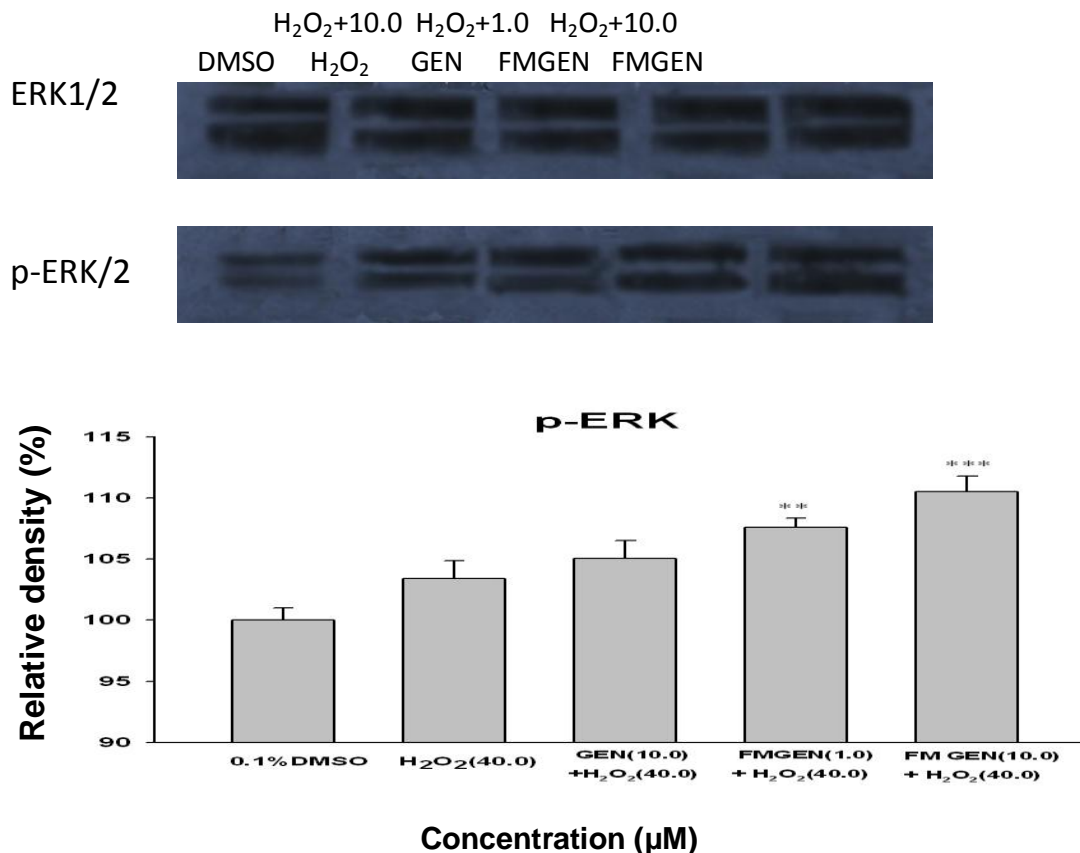


Figure 6. Western Blot analysis showing regulation of ERK1 / 2 protein expression in PC12 cells by H₂O₂. ** P < 0.01 versus genistein group, ***P < 0.001 versus genistein group).

104.3±1.2% and 93.3±1.8%, respectively. Results suggested that FMG could effectively down-regulate H₂O₂-induced expression of phosphorylation p38 of PC12 cells than genistein (Figure 7).

DISCUSSION

Many of the central nervous system diseases are closely related to oxygen free radicals, such as cerebral ischemia, Alzheimer's disease, Parkinson's disease, multiple sclerosis (Weber, 1994; Burton, 1995; Irani, 1997). PC12 cells are extremely similar to neurons in cell morphology, structure and function, so it has been widely used as a cell model for study of nerve cells (Saito et al., 2003). Numerous studies have shown that oxygen free radicals and their derivatives are closely related to cell apoptosis (Xiao-Qing et al., 2004; Li-Min, 2005). For example, some concentration of H₂O₂ can induce certain types of cells apoptosis (for example, PC12 cells, vascular endothelial cells, HL-60 cells), oxidized LDL can induce lymphocyte apoptosis, peroxy nitrite radicals can induce apoptosis of thymocytes and so on.

The LDH release is a classic indicator of cell function. If

LDH release increased, cell function was injured. Our study showed that FMG could decrease LDH release of PC12 cells injured by oxidative stress. It is probable that FMG could provide some protections against PC12 cells injury by oxidative stress.

MTT assay confirmed that cell growth and proliferation were suppressed when PC12 cells were treated with H₂O₂ for 24 h. However, FMG could effectively decrease the suppression. FCM with PI staining showed that FMG could reduce the H₂O₂-induced apoptosis of PC12 cells in a concentration dependent manner. It is further confirmed that FMG could provide protection against PC12 cells injury caused by oxidative stress.

This study showed that FMG could effectively reduce the H₂O₂-induced apoptosis of PC12 cells in a concentration dependent manner and have a better protective effect than the lead compound genistein. It is probable that FMG could provide protection against PC12 cells injury caused by oxidative stress. *In vitro* and *in vivo* experiments have confirmed that ERK1/2 kinase pathway is a signaling pathway of stimulating cell survival when cells were induced by H₂O₂ while the p38 kinase pathway is a signal transduction pathway of stimulating cell apoptosis when cells were induced by H₂O₂ (Inanami

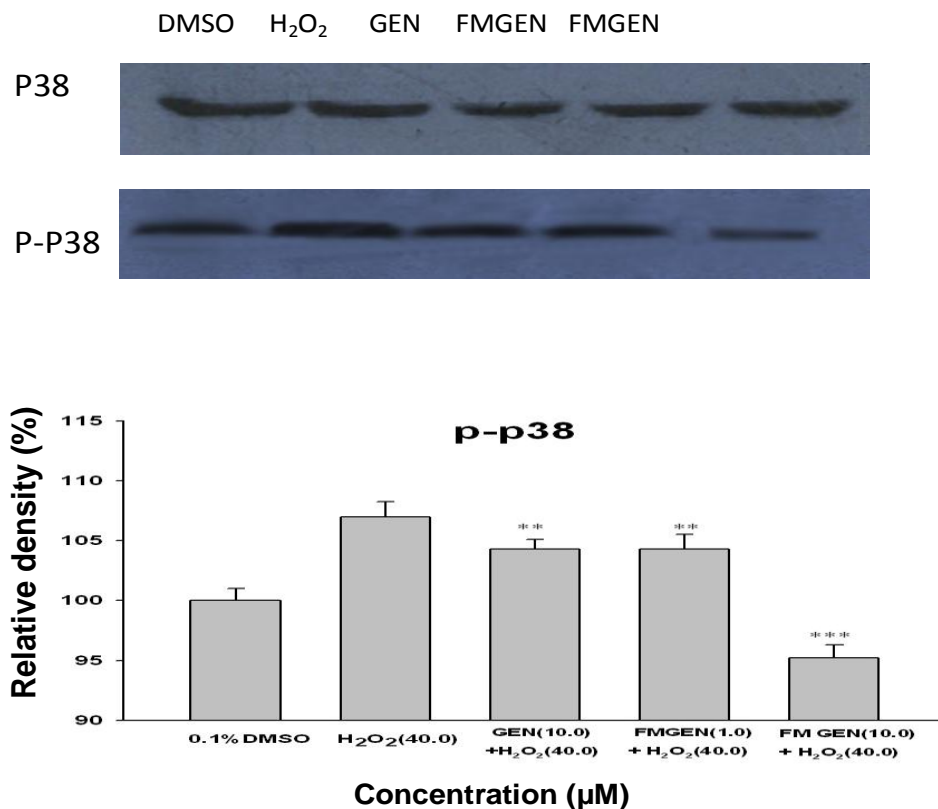


Figure 7. Western Blot analysis showing regulation of P38 protein expression in PC12 cells by H₂O₂. ** P < 0.01 versus solvent group,***P < 0.001 versus solvent group).

et al., 1999; Xia et al., 1995).

Recently, Lin et al reported that flavonoids could inhibit H₂O₂-induced cell death in human neuroblastoma SH-SY5Y cells (Lin et al., 2009; Niwa et al., 2002). Western Blot analysis results in our study confirmed that, FMG could activate phosphorylation of ERK1/2 of PC12 cells stronger than genistein. It is suggested that FMG might also protect against PC12 cells injury induced by H₂O₂ through increasing ERK1/2 phosphorylation.

The model of PC12 cells injury and apoptosis induced by H₂O₂ was established, using genistein as control group to test the activity of nine derivatives against oxidative stress injury in PC12 cells. Results in our study showed that FMG was the most effective article among test articles. In summary, FMG possesses the protective activity of PC12 cells injury by H₂O₂ *in vitro*. The protective activity of FMG on PC12 cells injury by H₂O₂ is possibly through up-regulating the expression of phosphorylation ERK1/2 and suppressing the activation of P38.

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